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Deregulation of *MUM1/IRF4* by chromosomal translocation in multiple myeloma

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The pathogenesis of multiple myeloma (MM), an incurable tumour causing the deregulated proliferation of terminally differentiated B cells, is unknown¹. Chromosomal translocations (14q1) affecting band 14q32 and unidentified partner chromosomes are common in this tumour, suggesting that they may cause the activation of novel oncogenes^{2,3}. By cloning the chromosomal breakpoints in an MM cell line, we show that the 14q+ translocation represents a t(6;14)(p25;q32) and that this aberration is recurrent in MM, as it was found in two of eleven MM cell lines. The translocation juxtaposes the immunoglobulin heavy-chain (*IgH*) locus to *MUM1* (multiple myeloma oncogene 1)/*IRF4* gene, a member of the interferon regulatory factor (IRF) family known to be active in the control of B-cell proliferation and differentiation. As a result, the *MUM1/IRF4* gene is overexpressed—an event that may contribute to tumorigenesis, as *MUM1/IRF4* has oncogenic activity *in vitro*. These findings identify a novel genetic alteration associated with MM, with implications for the pathogenesis and diagnostics of this tumour.

Chromosomal aberrations in the *IgH* locus on band 14q32 (14q+) are common in multiple myeloma (MM) (62% of cases) and represent translocations of a variety of loci into the *IgH* switch region^{4,5}. With the exception of some cases involving the *BCL-1* locus on chromosome 11q13 (ref. 6), the genes implicated in the partner chromosomes have not been identified. To investigate the nature of the sequences linked to the *IgH* locus in 14q+ chromosomes, we screened a number of cell lines for the presence of abnormally re-arranged *IgH* loci that could reflect the presence of chromosomal translocations. We used a Southern-blot hybridization assay aimed at identifying re-arranged *IgH* alleles in which *IgH* joining (JH) and constant (C) region (C μ , C γ or S γ ³, C α) sequences are not linked, as these alleles reflect illegitimate *IgH* switch recombinations that often represent chromosomal translocations⁷. When we used this assay, nine of eleven MM lines tested showed evidence of illegitimate switch recombinations (not shown)—a result consistent with a recent report using a similar assay⁵.

The pattern of *IgH* gene re-arrangements detectable in the SK-MM-1 cell line strongly suggested the presence of a chromosomal translocation (Fig. 1a). *Bam*HI digestion of SK-MM-1 genomic DNA and hybridization with a JH probe showed a restriction fragment (12.0 kb) that did not hybridize with any C

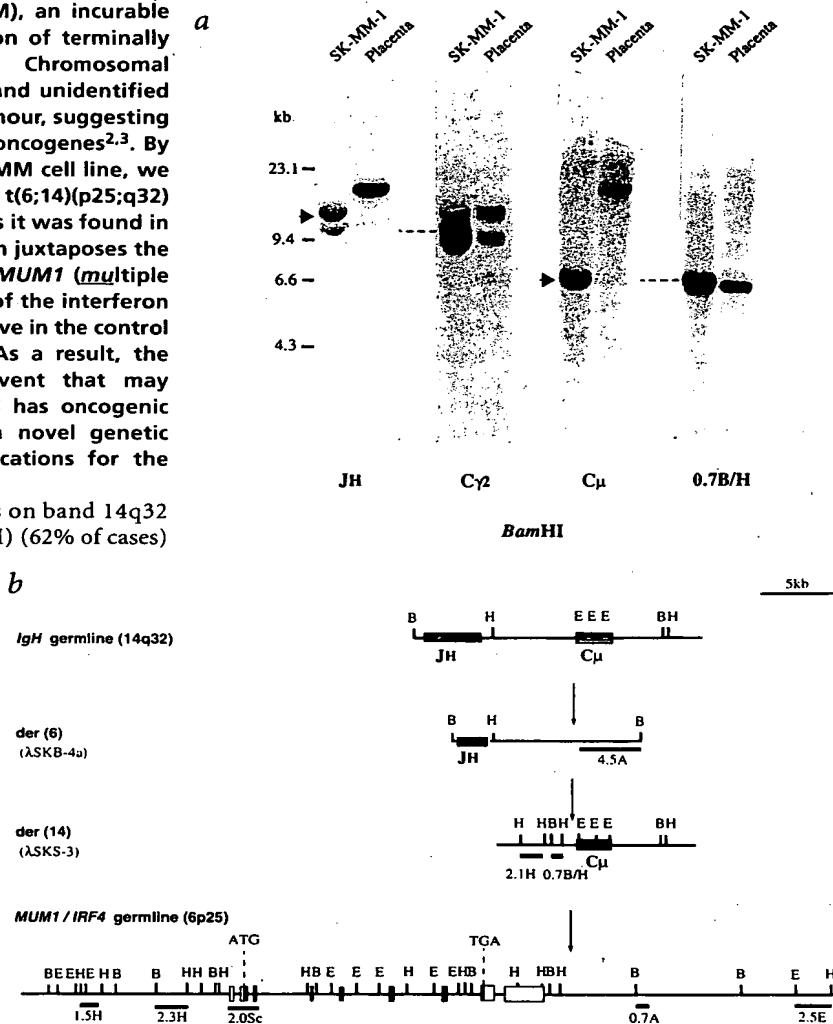


Fig. 1 Molecular cloning of 14q+ chromosome breakpoints in SK-MM-1 cells. **a**, Southern-blot analysis showing aberrant re-arrangements of the *IgH* locus. Arrowheads show rearranged non-reciprocally linked JH and C μ fragments. Co-migrating fragments (JH-C γ , left; C μ -0.7B/H, right) are linked by dashed lines. **b**, Schematic representation of the λSKB-4a and λSKS-3 clones, representing der(6) and der(14) breakpoints, and of the germline *IgH* and *MUM1* loci. The *MUM1* germline locus shows the genomic organization of the *MUM1* gene. Filled and empty boxes indicate coding and non-coding exons, respectively. The position and size of each exon, as well as their number, are approximate and have been determined by the pattern of hybridization of various cDNA probes, as well as by the presence of shared restriction sites in the genomic and cDNA sequences. Translation initiation (ATG) and stop (TGA) codons are indicated. Genomic probes used for Southern analysis are shown as solid bars below the map. Arrows indicate the position of the chromosomal breakpoint in the SK-MM-1 cell line. B, *Bam*HI; E, *Eco*RI; H, *Hind*III.

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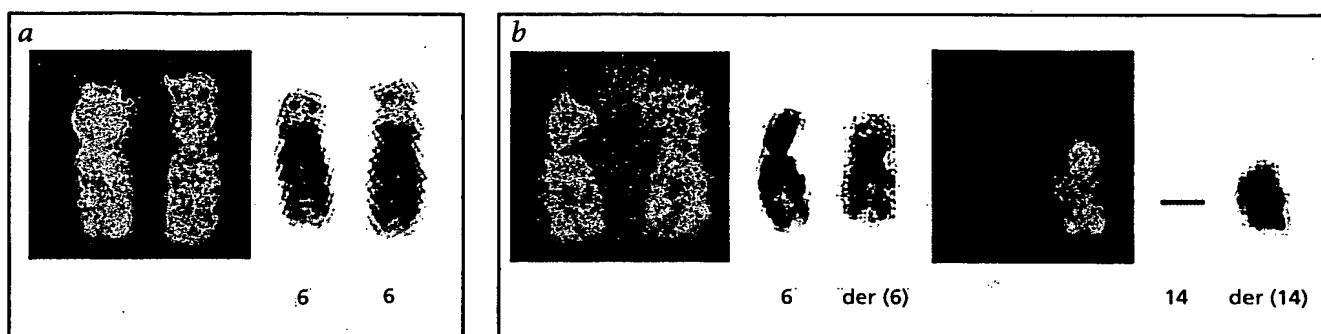


Fig. 2 FISH analysis of metaphase spreads derived from normal lymphocytes (a) and XG-7 cells (b). **a**, The *MUM1* locus mapped to chromosome 6p25 by FISH analysis using a 360-kb non-chimaeric YAC (y927E3). **b**, Splitting of YAC y927E3 signal to chromosome 6p25 and 14q32 in a metaphase spread from XG-7 cells. This cell line displays monosomy of chromosome 14. The right panel of each FISH picture displays the corresponding DAPI image, showing a G-banding-like stain.

probes, while a C μ probe identified a 6.5-kb fragment that did not contain any J sequences. The 12.0-kb and 6.5-kb fragments were considered potential chromosomal breakpoints and cloned from a phage library constructed from SK-MM-1 genomic DNA (clones λ SKB-4a; λ SKS-3 in Fig. 1b). A 0.7B/H probe from the λ SKS-3 phage insert representing non-*IgH* sequences (Fig. 1b) was used

to clone the corresponding normal locus from a library constructed from normal human DNA (Fig. 1b). This locus was assigned the name *MUM1* (*m*ultiple *m*yeloma oncogene 1; HUGO/GDB Nomenclature Committee). Restriction enzyme analysis (Fig. 1b) and partial nucleotide sequencing (not shown) suggested that the two SK-MM-1 phage inserts contain the chro-

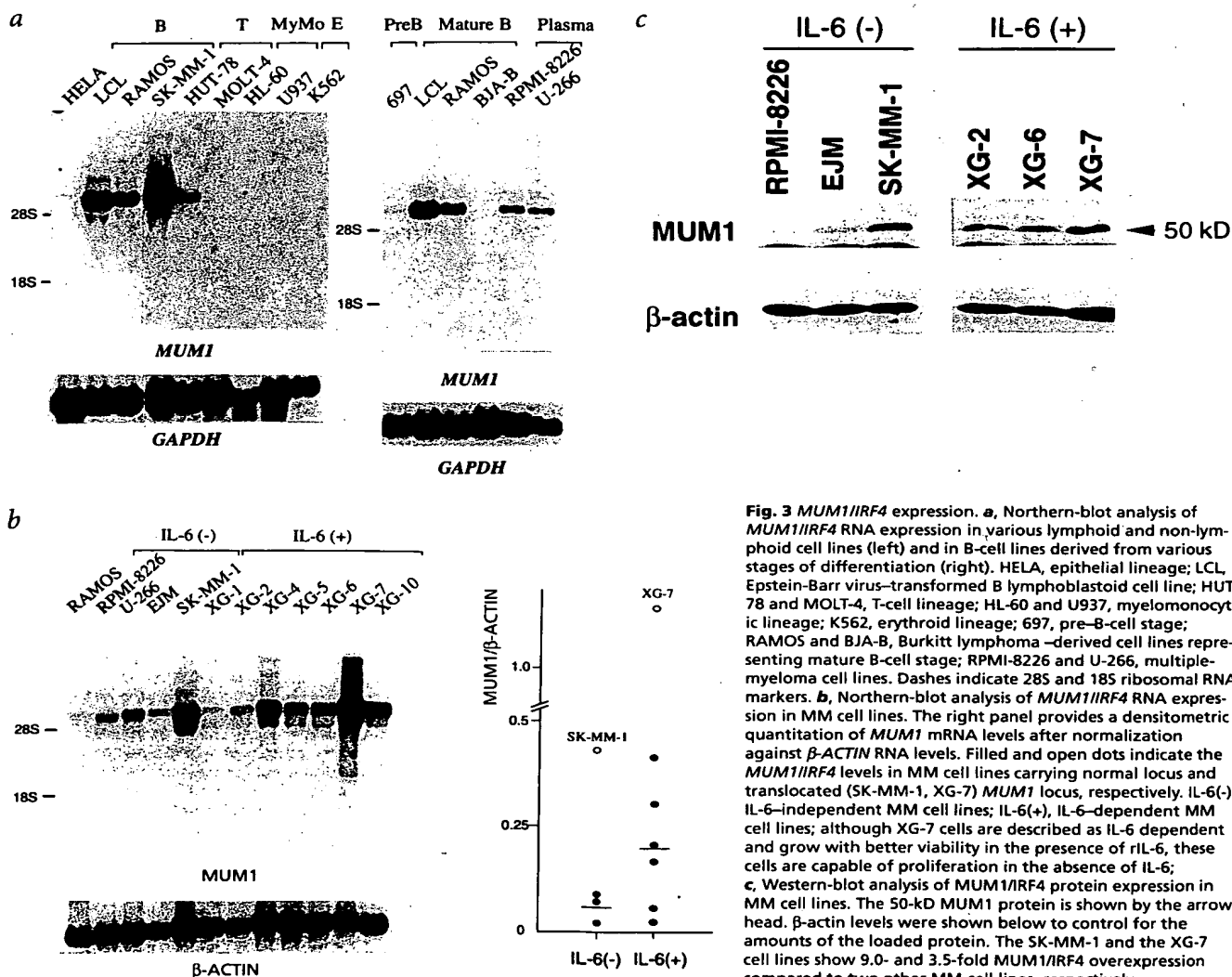


Fig. 3 *MUM1/IRF4* expression. **a**, Northern-blot analysis of *MUM1/IRF4* RNA expression in various lymphoid and non-lymphoid cell lines (left) and in B-cell lines derived from various stages of differentiation (right). HELA, epithelial lineage; LCL, Epstein-Barr virus-transformed B lymphoblastoid cell line; HUT-78 and MOLT-4, T-cell lineage; HL-60 and U937, myelomonocytic lineage; K562, erythroid lineage; 697, pre-B-cell stage; RAMOS and BJA-B, Burkitt lymphoma-derived cell lines representing mature B-cell stage; RPMI-8226 and U-266, multiple-myeloma cell lines. Dashes indicate 28S and 18S ribosomal RNA markers. **b**, Northern-blot analysis of *MUM1/IRF4* RNA expression in MM cell lines. The right panel provides a densitometric quantitation of *MUM1* mRNA levels after normalization against β -ACTIN RNA levels. Filled and open dots indicate the *MUM1/IRF4* levels in MM cell lines carrying normal locus and translocated (SK-MM-1, XG-7) *MUM1* locus, respectively. IL-6(-), IL-6-independent MM cell lines; IL-6(+), IL-6-dependent MM cell lines; although XG-7 cells are described as IL-6 dependent and grow with better viability in the presence of rIL-6, these cells are capable of proliferation in the absence of IL-6; **c**, Western-blot analysis of *MUM1/IRF4* protein expression in MM cell lines. The 50-kD *MUM1* protein is shown by the arrowhead. β -actin levels were shown below to control for the amounts of the loaded protein. The SK-MM-1 and the XG-7 cell lines show 9.0- and 3.5-fold *MUM1/IRF4* overexpression compared to two other MM cell lines, respectively.

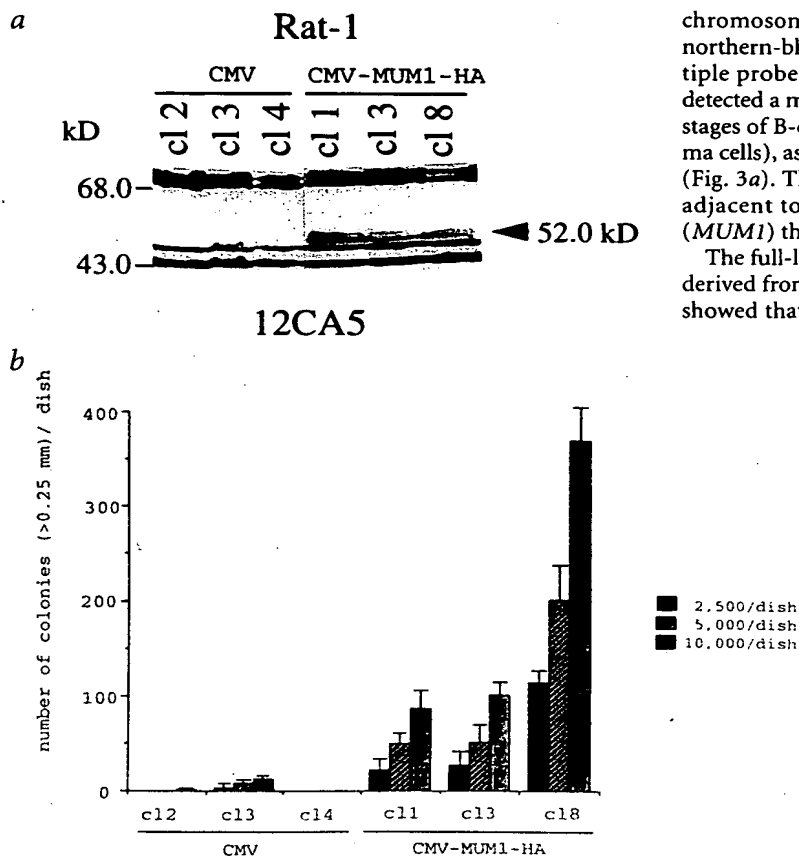


Fig. 4 *MUM1/IRF4* overexpression can transform Rat-1 fibroblasts *in vitro*. **a**, Western-blot analysis of *MUM1/IRF4*-transfected (CMV-MUM1-HA) and control-transfected (CMV) Rat-1 cell clones (c1) using the anti-HA (12CA5) MoAb. The 52-kD MUM1-HA protein is shown by an arrowhead. **b**, Anchorage-independent growth of Rat-1 cells expressing the exogenous MUM1-HA protein. The graph shows the number of colonies formed by each clone at three seeding densities (indicated at the right). Error bars indicate +1SD.

mosomal breakpoints of a reciprocal balanced translocation in the *IgH* and the *MUM1* loci.

To determine the chromosomal origin of *MUM1* sequences, a 360-kb non-chimaeric yeast artificial chromosome (YAC) committee (y927E3) spanning the *MUM1* breakpoint region was used as a probe in fluorescence *in situ* hybridization (FISH) analysis of metaphase spreads from normal human lymphocytes. This analysis indicated that *MUM1* is located on chromosome 6p25 (Fig. 2a); Southern-blot hybridization of the 2.1H and 4.5A probes (Fig. 1b) to DNA from somatic-cell hybrids representative of individual human chromosomes confirmed the mapping to chromosome 6 (not shown). The same YAC probe was then used to analyse the *MUM1* locus in eleven MM cell lines, including SK-MM-1. In SK-MM-1 cells (not shown), as well as in a second MM cell line (XG-7), the YAC hybridized to the 14q+ chromosome [der(14)] as well as to a der(6) chromosome (in addition to normal chromosomes 6) (Fig. 2b), whereas no abnormality was detectable in nine additional MM lines. Thus, the 14q+ chromosome was part of a reciprocal t(6;14)(p25;q32) in the SK-MM-1, while the FISH results strongly suggest the presence of an analogous aberration in XG-7 cells. These translocations were undetectable by conventional cytogenetic analysis because of the relatively small size of the telomeric region translocating from chromosome 6. This translocation is recurrent in MM, as it was detectable in two of eleven cases tested.

Next we investigated whether the *MUM1* locus adjacent to the

chromosomal breakpoints contained a transcriptional unit by northern-blot analysis of RNAs from various cell lines using multiple probes spanning the *MUM1* locus. Probe 2.1H (Fig. 1b) detected a major 6-kb RNA in B-cell lines representative of various stages of B-cell differentiation (from pre-B cells through to plasma cells), as well as in lines displaying a mature T-cell phenotype (Fig. 3a). This result indicates that 6p25 sequences immediately adjacent to the chromosomal breakpoints are part of a gene (*MUM1*) that is transcribed in lymphoid tissues.

The full-length *MUM1* cDNA was cloned from a cDNA library derived from normal human spleen and sequenced. This analysis showed that the predicted *MUM1* protein product was virtually identical to the *IRF4* gene (also called *ICSAT*, *PIP* or *LSIRF*), a member of the IRF family of transcription factors⁸⁻¹¹. This family includes at least six molecules that share homologous N-terminal DNA-binding domains and are active in the regulation of gene expression in response to signalling by interferons and by other cytokines¹²⁻¹⁵.

To determine the consequences of the translocation on the structure of the *MUM1/IRF4* gene, we used the *MUM1/IRF4* cDNA to determine the exon-intron organization of the corresponding genomic locus (Fig. 1b). This allowed us to determine that the chromosomal breakpoint was located 3' to the *MUM1/IRF4* gene in SK-MM-1 cells (Fig. 1b), indicating that the t(6;14) translocation had juxtaposed the switch region of *IgH* locus 3' to the *MUM1/IRF4* gene in the same transcriptional orientation. The entire *MUM1/IRF4* transcriptional unit appeared intact, suggesting that its coding potential is normal, though its regulation may be affected by *IgH* regulatory elements, particularly the late B-cell-specific transcriptional enhancer located 3' to C α locus¹⁶. Probes spanning 55 kb of the *MUM1* locus, including the *MUM1/IRF4* gene and 5' and 3' flanking sequences, failed to detect rearrangements in ten MM cell lines and eighteen MM cases studied (not shown).

These cell lines included the XG-7 line, which was shown to contain a t(6;14)(p25;q32) by FISH analysis (Fig. 2b), suggesting that, analogous to other translocations in *Ig* genes^{6,17}, the breakpoints of t(6;14)(p25;q32) can scatter along 6p25 at variable distances from the *MUM1* locus.

To investigate the functional consequences of chromosomal translocation on *MUM1/IRF4* expression, we compared the levels of *MUM1/IRF4* RNA in MM cell lines carrying t(6;14)(p25;q32) (SK-MM-1, XG-7) or lacking detectable 6p25 abnormalities. In general, the levels of *MUM1/IRF4* RNA expression tended to be higher (3.4-fold) in interleukin-6 (IL-6)-dependent than in IL-6-independent MM cell lines (Fig. 3b). However, SK-MM-1 and the XG-7 cell lines displayed the highest levels of *MUM1/IRF4* expression, a 7.5- and a 6-fold overexpression compared to the average of other MM cell lines (Fig. 3b). Immunoblot analysis of SK-MM-1 and XG-7 cell lines showed that increased RNA expression was associated with increased levels of *MUM1/IRF4* protein expression (Fig. 3c). Electrophoretic mobility shift assay (EMSA) of nuclear extracts using an IRF binding site as a probe (GBP-ISRE)¹⁰ showed that both cell lines carrying alterations of the *MUM1* locus (SK-MM-1 and XG-7) had higher amounts of DNA-bound MUM1/IRF4 than control MM lines (not shown). Collectively, these results indicated that the functional consequence of chromosomal translocation is the deregulation of *MUM1/IRF4* gene expression, leading to the production of increased amounts of functional MUM1/IRF4 protein.

To investigate whether *MUM1/IRF4* deregulation could contribute to malignant transformation, we tested whether transfection of a *MUM1/IRF4* expression vector in Rat-1 fibroblasts could increase their clonogenic properties in agar, a typical sign of malignant transformation. We transfected Rat-1 cells with an expression vector (CMV-MUM1-HA) in which a CMV promoter drives the expression of MUM1/IRF4 tagged by a haemagglutinin (HA) epitope recognizable from a specific monoclonal antibody. CMV-MUM1-HA transfected Rat-1 clones expressing detectable levels of exogenous MUM1-HA protein (Fig. 4a), but not control (CMV) transfected clones, acquired anchorage-independent growth in soft agar (Fig. 4b). These clones also displayed higher saturation density than transfected control clones during growth *in vitro* [$(1.52 \pm 0.31) \times 10^6 \text{ cm}^{-2}$ versus $(0.80 \pm 0.35) \times 10^6 \text{ cm}^{-2}$] (not shown). These results indicate that *MUM1/IRF4* can behave as an oncogene *in vitro* and support the notion that it may contribute to oncogenesis *in vivo*.

Several studies have shown that gene alterations commonly associated with most human tumours, such as inactivation of the *p53* tumour-suppressor gene and *RAS* oncogene mutations, can be found in MM^{18,19}. However, these lesions are found at low frequency and in association with advanced stages of the disease, suggesting that their role may be limited to tumour progression. Although in the present study the evidence of involvement of the *MUM1* locus is limited to MM cell lines, these alterations are associated with 14q+, a cytogenetic aberration often detectable at diagnosis²⁰. This suggests that *MUM1/IRF4* deregulation may be associated with early stages of MM development.

A role of *MUM1/IRF4* deregulation in oncogenesis is supported by its oncogenic activity *in vitro* (Fig. 4) and is consistent with the role of other *IRFs* as oncogenes and tumour-suppressor genes in various experimental systems^{21–24}. *MUM1/IRF4* has been shown to control the differentiation of B cells into plasma cells, as well as their proliferation in response to various mitogenic signals²⁵, suggesting that its deregulated expression may directly contribute to the aberrant phenotype of MM cells¹. Because its expression does not appear to be regulated by IFN^{8,10}, *MUM1/IRF4* may act as an effector of other cytokines that regulate B-cell development.

Methods

Cell lines. The phenotypic features of the MM cell lines used in this study (SK-MM-1, RPMI-8226, U-266, EJM, XG-1, XG-2, XG-4, XG-5, XG-6, XG-7 and XG-10) have been previously reported^{27–29}. The RPMI-8226 cell line was obtained through American Type Culture Collection. The SK-MM-1 and U-266 cell lines were gifts from A.N. Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY) and Dr. K. Nilsson (University of Uppsala, Uppsala, Sweden), respectively. The six XG cell lines (established in the laboratory of one of the authors, B.K.) were cultured in the presence of 1 ng/mL recombinant IL-6 as described previously²⁹.

Southern- and northern-blot analyses. Southern- and northern-blot analyses were performed as described previously¹⁷. The human *IgH* JH (6.6kb *Bam*HI-*Hind*III) and *IgH* Cμ (1.3-kb *Eco*RI) probes were provided by S.J. Korsmeyer. The human *IgH* Cγ2 probe (4.0-kb *Hind*III-*Bam*HI) was provided by V. Bertness. In Southern-blot analysis, high-molecular-weight DNA (10 μg) from SK-MM-1 cells or normal human placenta was completely digested with *Bam*HI, loaded on each lane and blotted to a nitrocellulose filter. The same filter was sequentially hybridized with the JH, Cμ, Cγ2 and 0.7B/H (Fig. 1b) probes. Total RNA (10 μg) was loaded on each lane for northern-blot analysis.

Genomic and cDNA library screening and DNA sequence analysis. Genomic libraries from SK-MM-1 and human placental DNAs were constructed and screened as described previously¹⁷. *MUM1* cDNA clones were isolated from an oligo-dT/random-primed cDNA library constructed from normal human spleen RNA (Clontech). DNA sequences were performed with a ABI 473A DNA sequencer (Applied Biosystems). Sequence homology searches were carried out through the BLAST e-mail server at the National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD.

YAC isolation and FISH analysis. PCR primers (sense 5'-TACTCGCAC-CTCTTGGCT-3'; antisense 5'-CTGGAGAGCAATGAACGG-3') derived from the 6p25 sequence within the last exon of the *MUM1* gene were used to screen a human CEPH YAC DNA pool (Research Genetics). Preparation of metaphase spreads, YAC DNA extraction and FISH analysis were performed as reported previously¹⁷.

Western-blot analysis. Cell pellets prepared from MM and Rat-1 cells were re-suspended in 1×Laemmli sample buffer and boiled for 5 min. Protein lysates derived from 10⁶ cells were fractionated on an SDS (7.5% for MM cells or 12.5% for Rat-1 cells) acrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The filter was blocked with 5% milk in Tris-buffered saline (TBS)-0.2% Tween and then incubated overnight at 4 °C with a 1/400 dilution of anti-ICSAT goat serum (M-17; Santa Cruz Biotechnology) or a 1/500 dilution of anti-HA monoclonal antibody (MoAb: 12CA5; Boehringer Mannheim) in TBS-0.1% Tween, 3% bovine serum albumin. This was followed by an incubation with anti-goat or anti-mouse IgG (1/3,000), horseradish peroxidase-linked antibody (Amersham) in TBS-0.1% Tween, 5% milk. Reactive bands were detected with an ECL system (Amersham).

Expression constructs. The *MUM1* cDNA, comprising a full-length ORF plus 96 and 0 bp of 5' and 3' untranslated regions, respectively, was linked in-frame to the COOH-terminal HA (MAYPYDVPDYASLGPGP) tag by PCR, blunt-ended by Klenow enzyme and cloned into blunt-ended *NofI* site of pHeBo-CMV eukaryotic expression vector³⁰.

Rat-1 cell transfection and soft-agar assay. Rat-1 cells plated at 1×10^6 per 100-mm dish in Dulbecco modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), were transfected with 3 pmol of pHeBo-CMV or pHeBo-CMV-MUM1-HA by a modified calcium phosphate method³⁰. Forty-eight hours after transfection, cells were reseeded into five 100-mm dishes and cultured in DMEM, supplemented with 10% FBS containing 600 μg/ml of G418. G418-resistant colonies were isolated after 10 days of selection. For clonogenicity analysis, Rat-1 cells were plated in triplicate at 2.5×10^3 , 5×10^3 and 1×10^4 cells per dish onto 35-mm agar plates containing DMEM, 10% FBS and 0.5% agar. After incubation for 2 weeks at 37 °C, 5% CO₂, colonies were scored when larger than 0.25 mm.

GenBank accession numbers. *MUM1* cDNA, U63738; *MUM1* 5' genomic region, U63739.

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